

Genetic deletion of β -arrestin 2 modulates LSD-stimulated behaviors in mice

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Research Article

Keywords: β Arr1, β Arr2, Lysergic acid diethylamide (LSD), β Arr2-KOs, MDL, LSD, head twitches, grooming, retrograde walking

Posted Date: May 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-544199/v1>

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Abstract

Recent evidence suggests that psychedelic drugs can exert beneficial effects on anxiety, depression, and ethanol and nicotine abuse in humans. However, their hallucinogenic side-effects often preclude their clinical use. Lysergic acid diethylamide (LSD) is a prototypical hallucinogen and its psychedelic actions are exerted through the 5-HT_{2A} serotonin receptor (5-HT_{2A}R). 5-HT_{2A}R activation stimulates Gq- and β -arrestin- (β Arr) mediated signaling. To separate these signaling modalities, we have used β Arr1 and β Arr2 mice. We find that LSD stimulates motor activities to similar extents in WT and β Arr1-KO mice, with non-significant effects in β Arr2-KOs. LSD robustly stimulates many surrogates of psychedelic drug actions including head twitches, grooming, retrograde walking, and nose-poking in WT and β Arr1-KO animals. By contrast, LSD slightly stimulates head twitches in β Arr2-KO mice, without effects on other surrogates. The 5-HT_{2A}R antagonist MDL100907 (MDL) blocks these LSD effects. LSD also disrupts prepulse inhibition (PPI) in WT and β Arr1-KOs; PPI is unaffected in β Arr2-KOs. MDL restores PPI in WT mice, but this antagonist is without effect and haloperidol is required in β Arr1-KOs. Collectively, these results reveal that LSD's psychedelic drug-like actions appear to require β Arr2.

Background

Lysergic acid diethylamide (LSD) is a prototypical psychedelic drug and is one of the most potent drugs in this class¹. LSD alters sensation, perception, thought, mood, sense of time and space, and consciousness of self in humans^{1,2}. Since LSD-induced states bear many similarities to early acute phases of psychosis² and because serotonin (5-HT) and LSD both contain an indolamine moiety, Woolley and Shaw³ proposed that aberrant 5-HT levels in brain may produce mental disturbances including psychosis. This suggestion gave rise to the 5-HT hypothesis for schizophrenia and stimulated researchers to study LSD in hopes of gaining a better understanding of the disorder. However, this research was largely curtailed when LSD was classified as a DEA Schedule I drug in the 1960's. Recent research has revealed that LSD has medicinal value in treating cluster headaches⁴, anxiety and depressive disorders in life-threatening conditions when combined with psychotherapy⁵, and it may have potential for studying human consciousness and substance abuse⁶⁻⁷.

LSD shares structural similarities to 5-HT¹. Thus, it is not surprising that LSD has high affinities for all thirteen 5-HT G protein coupled receptors (GPCRs)⁸⁻¹⁰. Besides 5-HT receptors, LSD activates other biogenic amine GPCRs⁸ and this polypharmacology may contribute to LSD's many actions. One activity in particular regarding LSD is its hallucinogenic actions. This activity is ascribed to 5-HT_{2A} receptor (5-HT_{2A}R) stimulation since in drug discrimination studies, potency is correlated with hallucinogenic potency in humans¹¹. Because the same psychedelics produce head twitches in mice, this response is used as a proxy for hallucinations in humans¹², even though non-psychedelic drugs like 5-hydroxytryptophan induce robust head-twitch responses (HTRs)¹³. Hallucinogen-induced HTRs in rodents are blocked by the highly selective 5-HT_{2A}R antagonist MDL100907¹⁴⁻¹⁶ and are absent in *htr2A*

knockout (KO) mice¹⁷⁻¹⁸. In addition, human studies have shown the hallucinogenic actions of LSD are blocked with the 5-HT_{2A}R preferring antagonist ketanserin¹⁹. Thus, the hallucinogenic effects of LSD appear to be mediated through the 5-HT_{2A}R²⁰.

The 5-HT_{2A}R is a rhodopsin family member of GPCRs that is coupled to G_q protein and β -arrestin (β Arr) mediated signaling²¹⁻²⁴. Recent experiments reveal the 5-HT_{2A}R preferentially activates G_q family members, with moderate activity at G_z, and minimal activities at G_{i1}, G_{12/13}, and G_s-family members²⁵. However, the 5-HT_{2A}R binds to both β Arr1 and β Arr2 proteins *in vitro* and is complexed with these β ArRs in cortical neurons *in vivo*²⁴. While most GPCR agonists, like 5-HT, activate both G protein and β Arr signaling, ligand binding can activate also G protein-dependent signaling while serving to activate or inhibit β Arr-mediated signaling. Hence, a given ligand can act as an agonist at one pathway while inhibiting the other pathway or it can possess combinations of these actions. This property is termed functional selectivity or biased signaling²⁶⁻²⁸ and ligands have been developed to exploit these signaling features²⁹. Although LSD activates G protein signaling at many GPCRs¹⁰, this psychedelic stimulates β Arr-mediated responses at most tested biogenic amine GPCRs⁸. Interestingly, LSD displays β Arr-biased signaling at the 5-HT_{2A}R^{9-10,25}. Most 5-HT_{2A}R-containing neurons express both β Arr1 and β Arr2²⁴, and global β Arr1 and β Arr2 knockout (KO) mice have been generated³⁰⁻³¹. Since LSD is β Arr biased at the 5-HT_{2A}R, the present investigations were conducted to determine whether LSD produces behavioral effects that were differential among the wild-type (WT) and β Arr1-KO, and WT and β Arr2-KO mice.

Results

Effects of *Arb1* or *Arb2* deletion on LSD-stimulated motor activities. LSD has been reported to stimulate, inhibit, or produce biphasic effects on a variety of motor activities in rodents^{17,32-36}. We examined responses to LSD in the global β Arr1-KO and global β Arr2-KO mice to determine whether disruption of either gene product could modify the behavioral responses to this hallucinogen and to test whether 5-HT_{2A}R antagonism could block these effects. Locomotor, rearing, and stereotypical activities were monitored at 5-min intervals over the 120 min test in both the β Arr1 and β Arr2 genotypes (Supplementary Figs. S1-S2).

When cumulative baseline locomotion was examined in β Arr1 mice, activity was not differentiated by genotype or by the pre-assigned treatment condition (Supplementary Table S1). Following LSD injection, only treatment effects were found (Fig. 1a). Here, locomotor activities were stimulated by LSD relative to control groups given the vehicle or 0.5 mg/kg MDL alone (p -values \leq 0.001). When administered with LSD, both doses of MDL blocked the locomotor-stimulating effects of this psychedelic (p -values \leq 0.001). It should be emphasized that no sex effects were detected in any experiments in this manuscript.

An examination of cumulative baseline rearing and stereotypical activities in the β Arr1 mice found these responses to be significantly lower in some pre-assigned treatment groups than in others (p -values \leq 0.001) (Supplementary Table S1). To correct for these baseline differences in the subsequent

LSD-post injection analyses, the rearing and stereotypical data were submitted separately to ANCOVA. No significant effects of LSD were observed for rearing (Fig. 1b). By comparison for stereotypical activities, ANCOVA revealed a significant treatment effect in β Arr1 mice following LSD administration ($p=0.024$). Nevertheless, Bonferroni *post-hoc* analyses only identified a trend between the group treated with LSD and the group given MDL alone ($p=0.062$) (Fig. 1c). Collectively, these results indicate that LSD stimulates locomotor activities to similar extents in the WT and β Arr1-KO animals, and the 5-HT2AR antagonist blocks these responses. Rearing and stereotypical activities are unaffected by LSD in either genotype

When baseline motor activities were evaluated in the β Arr2 mice, no significant differences were found (Supplementary Table S2). Effects of LSD in the β Arr2-KO mice were quite different from those of the WT animals. LSD was more potent in stimulating cumulative locomotor activities in the WT than in the β Arr2-KO mice (p -values <0.001) (Fig. 2a). When locomotion was analyzed within WT animals, the LSD-stimulated responses were higher than those in the vehicle and MDL controls, as well as in the treatment groups administered MDL with LSD (p -values <0.001). Hence, all three doses of the 5-HT2AR antagonist were efficacious in suppressing the LSD-induced hyperlocomotion. Although LSD increased locomotor activity in β Arr2-KO mice, it was not significantly different from any other treatment group.

Similar to locomotion, LSD also stimulated rearing activities to a greater extent in WT compared to β Arr2-KO mice (p -values <0.001) (Fig. 2b). In WT animals, rearing activities were increased with LSD over that of the vehicle and MDL controls (p -values <0.001). When 0.1 or 0.5 mg/kg MDL was given with LSD, both doses reduced the LSD-stimulated rearing activities to control levels (p -values ≤ 0.001). By comparison, LSD was without effect in the β Arr2-KO mice.

An assessment of stereotypical activities failed to find any genotype differences between the β Arr2 mice (Fig. 2c). Nonetheless, treatment effects were evident with LSD stimulating stereotypical activities over that of the vehicle and MDL controls (p -values ≤ 0.013). Notably, 0.5 mg/kg MDL abrogated the LSD effects ($p=0.003$). Together, these results indicate that LSD stimulates motor responses to similar extents in the WT β Arr1 and WT β Arr2 mice, and in the β Arr1-KO animals. The 5-HT2AR antagonist blocks these LSD-stimulated activities. By striking comparison, LSD exerts minimal effects on these same responses in the β Arr2-KO mice where none of their motor activities were significantly increased above that of controls.

LSD effects on additional behaviors. LSD modifies a number of behaviors in mice^{12,17,37-41} that include, at least, HTRs, grooming, and retrograde walking. When these responses were examined in the β Arr1 mice, no genotype differences were noted, although overall treatment effects were evident. Relative to the vehicle and MDL controls, LSD stimulated HTRs in the WT and β Arr1-KO mice (p -values <0.001) (Fig. 3a). When 0.1 or 0.5 mg/kg MDL was administered with LSD, both doses of the 5-HT2AR antagonist blocked the LSD effects by restoring the numbers of HTRs to those of controls. Aside from HTRs, LSD augmented also grooming over that of the controls (p -values <0.001) (Fig. 3b). When 0.1 or 0.5 mg/kg MDL was given

with LSD, both doses of the 5-HT_{2A}R antagonist normalized the LSD stimulatory effects to those of controls (p -values <0.001).

Besides HTRs and grooming, LSD was efficacious in potentiating retrograde walking in the WT and β Arr1-KO mice compared to the vehicle and MDL controls (p -values <0.001) (Fig. 3c). With LSD, both 0.1 and 0.5 mg/kg MDL depressed retrograde walking (p -values <0.001). Nose poking behaviors were examined also. Here, LSD increased nose-poking over that of controls (p -values <0.001) (Fig. 3d). When MDL was administered with LSD, both doses of the 5-HT_{2A}R antagonist normalized the LSD stimulated nose-poking behaviors (p -values <0.001).

In contradistinction to β Arr1 mice, genotype differences were identified between the β Arr2 animals. HTRs were significantly increased with LSD in WT relative to β Arr2-KO mice ($p<0.001$) (Fig. 4a). Genotype effects were noted also in the 0.05 mg/kg MDL plus LSD group ($p<0.001$). In WT mice, HTRs were stimulated by LSD and they were still enhanced when 0.05 MDL was given with LSD relative to the vehicle and MDL controls (p -values <0.001). Notably, both 0.1 and 0.5 mg/kg MDL significantly reduced the LSD-stimulated responses (p -values ≤ 0.002) – with the higher MDL dose being the more efficacious in suppressing HTRs to control levels ($p<0.001$). In the β Arr2-KO mice, the LSD (p -values ≤ 0.023) and 0.05 mg/kg MDL plus LSD treatments (p -values ≤ 0.006) increased HTRs compared to the vehicle and MDL controls. Only 0.5 mg/kg MDL was sufficient to normalize this LSD-stimulated response in the β Arr2-KO mice ($p=0.019$).

For grooming, the durations of responding were higher in WT than in the β Arr2-KO groups administered LSD alone, 0.05 mg/kg MDL plus LSD, or 0.5 mg/kg MDL with LSD (p -values ≤ 0.016) (Fig. 4b). In WT mice, LSD augmented grooming relative to the vehicle and MDL controls ($p<0.001$). While 0.05 mg/kg MDL failed to block the LSD effects, both of the 0.1 and 0.5 mg/kg doses were efficacious in normalizing the responses (p -values <0.001). In β Arr2-KO animals, the duration of grooming to LSD was not significantly different from the vehicle and MDL controls. Nevertheless, grooming was enhanced in the group administered 0.05 mg/kg MDL plus LSD compared to all groups (p -values ≤ 0.013), except those given LSD alone.

Since LSD can induce alterations in tactile perception⁴², we examined grooming in detail as it has a chained organization of responses in rodents⁴³. Note, that since the WT β Arr1 and WT β Arr2 mice responded identically to the different treatment conditions, only one of the WT strains is represented. Analyses of the video-recordings confirmed that all genotypes engaged in a normal sequence of grooming beginning with the face, progressing down the body, and ending at the feet or tail (Movie 1). When LSD was administered, the sequence of grooming in the WT and β Arr1-KO mice became abbreviated, non-sequential, and/or restricted to one area of the body (Movies 2-3). By sharp comparison, the grooming sequence was complete and rarely perturbed in the β Arr2-KO animals (Movie 4). When the 5-HT_{2A}R antagonist MDL was administered alone, the organization of grooming was intact in the WT and β Arr1-KO mice (Movie 5). By comparison, with MDL the β Arr2-KO animals often paused in grooming bouts and/or displayed twitching of the neck and back muscles; however, they would finish the grooming

sequence (Movie 6). The patterns of grooming among the genotypes administered MDL plus LSD were divergent. In WT mice given MDL plus LSD, the organization of grooming was restored (Movie 7). When the β Arr1 mutants received the same treatment, they began the grooming sequence, engaged in focal grooming of a part of the body, and then completed the sequence (Movie 8). When this same drug combination was administered to β Arr2-KO mice, they usually began the sequence appropriately, but at some mid- or later-point they would become focused on one area of grooming (Movie 9). Nevertheless, they usually completed the grooming sequence

Aside from abnormalities in the organization of grooming, LSD also induced retrograde walking and stimulated nose-poking behaviors. No significant genotype effects were obtained for retrograde walking (Fig. 4c). In WT mice, LSD potentiated the incidences of retrograde walking compared to the MDL and vehicle controls ($p < 0.001$). Although 0.05 mg/kg MDL was ineffective in decreasing this LSD-stimulated behavior, both 0.1 and 0.5 mg/kg MDL suppressed this response (p -values < 0.001). By contrast, LSD was without any significant effect on retrograde walking in the β Arr2-KO animals. Similar to retrograde walking, no genotype effects were observed for nose poking behavior (Fig. 4d). In WT mice, LSD stimulated nose-poking behaviors relative to all other groups (p -values < 0.001). All doses of the 5-HT2AR antagonist reduced the LSD-stimulated nose poking to the levels of the vehicle and MDL controls. No treatment effects were noted among the β Arr2-KO animals.

In summary, responses to LSD across these LSD-stimulated behaviors were similar between the β Arr1 genotypes and the 5-HT2AR antagonist reduced these responses to levels of the vehicle and MDL controls. Importantly, the WT mice responded quite differently than the β Arr2-KO animals. HTRs and grooming to LSD were significantly higher in WT than in β Arr2-KO mice. LSD did not significantly increase retrograde walking or nose poking behaviors in the β Arr2-KO animals. Notably, LSD disrupted the sequences of grooming in WT and in β Arr1-KO mice; the β Arr2-KO animals were unaffected. Nonetheless, divergent responses to MDL alone or MDL plus LSD were observed among the genotypes, indicating actions required by 5-HT2AR activation.

LSD and MDL100907 effects on prepulse inhibition. LSD disrupts PPI in both rats and humans and the response can be restored with 5-HT2AR antagonists^{37,44}. β Arr1 mice were pre-treated with the vehicle or with 0.1 or 0.5 mg/kg MDL as controls. Subsequently, they were administered the vehicle or 0.3 mg/kg LSD and tested in PPI. No significant genotype or treatment effects were observed for null activity or in response to the 120 dB startle stimulus (Supplementary Fig. S3a-b). In contrast, genotype effects were found in PPI where 0.1 and 0.5 mg/kg MDL normalized PPI in WT mice, whereby these same doses were ineffective in the β Arr1-KO animals (p -values ≤ 0.018) (Fig. 5a). As anticipated, LSD depressed PPI in both β Arr1 genotypes relative to their MDL and vehicle controls (p -values ≤ 0.002). Thus, LSD depressed PPI in both WT and β Arr1-KO mice, while MDL only restored PPI in WT animals.

Since haloperidol can normalize PPI in mouse models⁴⁵, we tested whether this antipsychotic drug could normalize the LSD-disrupted PPI in the β Arr1-KO mice. For null activity, no genotype effects were evident (Supplementary Fig. S3c). Overall treatment effects were found in the β Arr1 animals where null activities

were higher in the 0.1 mg/kg haloperidol plus LSD group than in mice treated with the vehicle or haloperidol alone (p -values \leq 0.009). An assessment of startle activity revealed that responses were lower overall in the WT relative to β Arr1-KO mice (p =0.028) (Supplementary Fig. S3d). For PPI, responses were reduced overall in the β Arr1-KO compared to the WT animals (p =0.008) (Fig. 5b). Treatment effects were observed also, where LSD suppressed PPI relative to all other treatment conditions (p -values \leq 0.002). Here, haloperidol normalized the LSD-disrupted PPI in both WT and β Arr1-KO mice.

PPI responses in the β Arr2 mice were examined also. No significant genotype effects were reported for null or startle activities. Overall null activity was decreased in the 0.1 mg/kg MDL plus LSD group compared to the vehicle control and the LSD group (p -values \leq 0.003) (Supplementary Fig. S4a). No significant effects were detected for startle activity (Supplementary Fig. S4b). Nevertheless, striking genotype differences were evident for PPI (Fig. 6). Here, responses to LSD and to the 0.05 MDL plus LSD treatments were reduced in WT relative to the β Arr2-KO mice (p -values \leq 0.001). In WT animals, LSD suppressed PPI compared to the MDL and vehicle controls (p =0.001). PPI was normalized when 0.1 mg/kg MDL was given with LSD. By dramatic comparison, LSD was completely without effect in the β Arr2-KO mice. Collectively, these findings show that LSD disrupts PPI in both genotypes of the β Arr1 mice. PPI was aberrant also the WT β Arr2 animals. The 5-HT_{2A}R antagonist restored PPI in both WT strains, whereby haloperidol was required to normalize it in β Arr1-KO mice. By contrast, PPI in β Arr2-KO mice was unaffected by LSD.

Effects of *Arb1* or *Arb2* deletion on 5-HT_{2A}R expression. We examined whether deletion of *Arb1* or *Arb2* could alter 5-HT_{2A}R expression by radioligand binding with brains from WT and β Arr1-KO, and WT and β Arr2-KO littermates. When [³H]-ketanserin competition binding was examined, displacement with DOI and K_i values were found to be very similar with membranes from the WT and β Arr1-KO and the WT and β Arr2-KO brains (Fig. 7a). We examined also 5-HT_{2A}R immunofluorescence in β Arr1 and β Arr2 brain sections (Fig. 7b-e). Here, we detected no apparent alterations in the relative receptor distributions among the genotypes. Together, these results are consistent with the hypothesis that neither global *Arb1* nor global *Arb2* genetic deletion decreases 5-HT_{2A}R expression.

Discussion

In the present study, we analyzed whether global deletion of *Arb1* or *Arb2* was involved in LSD-stimulated responses in mice. In many cases, we found that LSD modified behaviors in both strains of WT mice, as well as in the β Arr1-KO animals. By contrast, LSD-induced responses in the β Arr2-KO animals were either minimal or non-existent. Collectively, these results suggest the LSD-stimulated responses require β Arr2. In this regard, β Arr2 is reported to play a similar role in morphine-stimulated hyperlocomotion⁴⁶ and amphetamine-stimulated locomotor and rearing activities in β Arr2 mice⁴⁷.

While we found LSD stimulates locomotion in mice, in rats it has been reported to decrease ambulation³⁵ or increase locomotion^{32,33,36}. While an inhibitory response to 0.2 mg/kg LSD was observed in rats, we only saw stimulatory effects with 0.3 mg/kg LSD and in pilot studies, doses of 0.1 to 0.5 mg/kg LSD

were all stimulatory. An absence of LSD inhibitory effects could be attributed to differences in species tested, test environment and apparatus, and/or test procedure. For instance, in humans LSD's behavioral effects can be context specific^{1,2} and our 30 min habituation to the open field prior to LSD administration may have reduced emotionality in our mice, such that only the stimulatory effects of LSD were evident.

To determine whether the locomotor-stimulating effects of LSD were due to 5-HT_{2A}R activation, MDL was used as an antagonist. When used alone, this antagonist exerted no effects on motor performance in either β Arr mouse strain. Importantly, 0.1 and 0.5 mg/kg MDL blocked the locomotor-stimulating effects of LSD in both WT strains and in the β Arr1-KO animals. A similar effect has been observed in rats³⁶. Hence, the present results indicate that the LSD-induced hyperactivity in β Arr mice is promoted through the 5-HT_{2A}R.

Besides motor activity, we examined the effects of LSD on HTRs, grooming, retrograde walking, and nose-poking behaviors. LSD and other psychedelics are well-known to stimulate HTRs in mice^{17,38,41} and this behavior has been proposed as a proxy for hallucinations in humans¹². Compared to vehicle, LSD stimulated HTRs to similar extents in WT and β Arr1-KO mice. In β Arr2-KO animals, this response was severely blunted compared to the WT controls. These results were unexpected since the individual competition binding curves could be superimposed among the different genotypes. Regardless, in both β Arr1 and β Arr2 mice, MDL reduced HTRs to levels of the vehicle controls. These findings are consistent not only with the known action of MDL on blocking HTRs to various hallucinogens¹⁴⁻¹⁶, but also on the inability of LSD and other psychedelics to induce this response in the *htr2A* homozygous mutant mice^{17,18,38}.

Aside from HTRs in rodents, LSD accentuates grooming behaviors in cats⁴⁸ and it can stimulate or inhibit grooming in mice^{39,40}. In our investigations, LSD augmented grooming in both WT strains, and in β Arr1-KO animals. By comparison, this psychedelic was ineffective in β Arr2-KO mice. In both WT strains and in β Arr1-KO animals, 0.1 and 0.5 mg/kg MDL returned the LSD-stimulated grooming to control levels. Thus, antagonism of the 5-HTR_{2A} was sufficient to restore LSD-induced grooming to baseline.

Effects of LSD were examined also for the organization of grooming behavior. Under vehicle treatment, all mice displayed similar patterns of grooming that began with the face, progressed to the flanks, and ended with the feet or tail. LSD disturbed this sequence of events in both WT strains and in β Arr1-KO mice. By comparison, grooming in the β Arr2-KO mice was largely unaffected by LSD. MDL did not alter grooming in the WT and β Arr1-KO mice, whereas it prolonged grooming and promoted twitching of the neck and back muscles in β Arr2-KO animals. This 5-HT_{2A}R antagonist blocked the LSD-disrupting effects on the organization of grooming in WT mice and it mostly restored it in β Arr1-KO animals. The MDL-LSD combination in β Arr2-KO animals produced some disturbances, but the mice typically completed the grooming sequence. Together, these results suggest that additional receptor systems may be involved in the LSD-induced grooming responses.

LSD effects on retrograde walking and nose-poking responses were examined also. We found LSD to stimulate these behaviors in WT animals from both strains, as well as in the β Arr1-KO mice. However, LSD promoted neither response in β Arr2-KO animals. Nevertheless, in the other genotypes MDL restored retrograde walking and nose-poking to the levels of vehicle controls. Hence, this 5-HT2AR antagonist normalized these LSD-stimulated behaviors.

LSD-induced states share many similarities with the early acute phases of psychosis². PPI is abnormal in individuals diagnosed with schizophrenia⁴⁹ and LSD disrupts PPI in rats^{36,39,44}. In β Arr1 mice, LSD impaired PPI in both genotypes without affecting startle or null activities. Both 0.1 and 0.5 mg/kg MDL restored the LSD-disrupted PPI, but only in WT mice; an effect consistent with the action of the 5-HT2AR antagonist MDL11,939 in rats⁴⁴. By comparison, MDL was ineffective in blocking the LSD effects in β Arr1-KO animals. Since LSD activates human dopamine D2 receptors^{8,50}, we used haloperidol as a D2 antagonist. We found this antagonist to restore the LSD-disrupted PPI in the β Arr1-KO mice. Parenthetically, both 0.1 and 0.2 mg/kg haloperidol failed to rescue PPI in rats given 0.1 mg/kg LSD (s.c.)³⁶; the possible reasons for this discrepancy in mice versus rats are unclear. When β Arr2 mice were tested, LSD disrupted PPI selectively only in WT mice. Notably, β Arr2-KO mice were completely unresponsive to this psychedelic. As with WT animals from the β Arr1 strain, MDL also normalized the LSD-disrupted PPI in the WT β Arr2 mice. Thus, the LSD effects on PPI in the β Arr mice are complex, with restoration of PPI with MDL in both strains of WT mice, normalization of PPI with haloperidol in β Arr1-KO animals, and without any discernable effect in β Arr2-KO subjects.

LSD and other psychedelics are well-known for their hallucinogenic actions¹ and these responses have been attributed to 5-HT2AR agonism¹¹. We observed LSD to stimulate motor activity, head twitches, grooming, retrograde walking, and nose-poking in both strains of WT mice and in β Arr1-KO animals. LSD also disrupted PPI in these same genotypes. The LSD-elicited responses in β Arr2-KO mice were either significantly attenuated or completely absent. In conditions where LSD produced changes in behavior, these alterations were blocked with the 5-HT2AR antagonist MDL. While these results suggest that the 5-HT2AR is an essential component for all these responses, it should be recalled that LSD exerts a plethora of actions at many GPCRs⁸⁻¹⁰ and, aside from HTRs, other behaviors are inconsistently affected by hallucinogens¹⁷. Hence, it is possible that LSD's effects on the 5-HT2AR are involved in a cascade of many GPCR-signaling events mediating these varied responses.

Like other GPCRs, agonist actions at the 5-HT2AR can lead to G protein-dependent and -independent signaling, the latter of which involves β Arr²²⁻²⁴. While both β Arr1 and β Arr2 are expressed ubiquitously in adult rodent brain, expression of β Arr2 mRNA is much higher than that for β Arr1—except in selected brain areas⁵¹. Thus, it may be surprising that the LSD-elicited responses were less disturbed in the β Arr1-KO than in the β Arr2-KO mice, because in the β Arr1-KO animals β Arr2-mediated signaling is still retained. In this regard, it is especially intriguing that LSD-induced HTRs were much more robust in both WT strains and in the β Arr1-KO animals, than in the β Arr2-KO mice. Our results with LSD suggest that β Arr2 may be essential for the expression of hallucinogenic-like actions at the 5-HT2AR.

Methods

Subjects. Adult male and female WT and β Arr1-KO, and WT and β Arr2-KO mice were used in these experiments³⁰⁻³¹. All mice had been backcrossed onto a C57BL/6J genetic background. Heterozygotes were used to generate the respective WT and KO animals. The mice were housed 3-5/cage in a temperature- and humidity-controlled room on a 14:10 h (lights on at 0600 h) light-dark cycle with food and water provided *ad libitum*. All experiments were conducted with an approved protocol from the Duke University Institutional Animal Care and Use Committee and all experiments and methods were performed in accordance with the relevant regulations and ARRIVE guidelines.

Drugs. The drugs consisted of (+)-LSD-(+)-tartrate (NIDA Drug Supply Program, Bethesda, MD), MDL 100907 (Bio-Techne Corp., Minneapolis, MN), haloperidol (Sigma-Aldrich, St. Louis, MO), and (-)-1-(2,5-diethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI; Sigma-Aldrich). The vehicle was composed of N,N-dimethylacetamide (final volume 0.5%; Sigma-Aldrich) and brought to volume with 5% 2-hydroxypropoyl- β -cyclodextrin (Sigma-Aldrich) in water (Mediatech Inc., Manassas, VA). All drugs were administered (i.p.) in a 5 mL/kg volume. All studies used groups that were administered the vehicle and the 5-HT_{2A}R antagonist, MDL100907 as controls.

Open field activity. Motor activities were assessed in an open field (21 x 21 x 30 cm; Omnitech Electronics, Columbus, OH) illuminated at 180 lux⁴⁵. All behaviors were filmed. Mice were injected with the vehicle or different doses of MDL and placed into the open field. Thirty min later, they were administered the vehicle or LSD and were immediately returned to the open field for 90 min. Motor activity was monitored using Fusion Integra software (Omnitech) for locomotor activity (distance traveled), rearing (vertical beam-breaks), and stereotypical activities (repetitive beam-breaks less than 1 sec) in 5-min blocks or as cumulative activities.

Head twitch, grooming, and retrograde walking. These behaviors were filmed during assessment of motor activity. The responses were scored by blinded observers over the first 30 min following injection of the vehicle or LSD after collection of baseline activity. The data are expressed as the numbers of head twitches, duration of grooming, and incidences of retrograde walking.

Nose-poking responses. Nose-pokes were monitored in a 5-choice serial reaction-time apparatus (Med Associates Inc., St. Albans, VT)⁵². Each chamber had five LED-illuminated 1.24 cm² nose-poke apertures with infrared diodes to register nose pokes. No food or liquid reward was available. Mice were injected with the vehicle or different doses of MDL and returned to their home-cages. Thirty min later, the animals were injected with the vehicle or different doses of LSD and were placed immediately into the operant chambers for 30 min. The data are depicted as the numbers of head pokes.

Prepulse inhibition (PPI). PPI of the acoustic startle response was conducted using SR-LAB chambers (San Diego Instruments, San Diego, CA) as reported⁴⁵. Mice were injected with vehicle or different doses of MDL or with 0.1 mg/kg haloperidol and returned to their home cages. Fifteen min later the animals were given the vehicle or different doses of LSD and were placed into the apparatus. After 10 min of

habituation to a white noise background (64 dB), testing began. Each test consisted of 42 trials with 6 null trials, 18 pulse-alone trials, and 18 prepulse-pulse trials. Null trials comprised the white noise background, pulse trials consisted of 40 ms bursts of 120 dB white-noise, and prepulse-pulse trials were composed of 20 ms pre-pulse stimuli that were 4, 8, or 12 dB above the white-noise background (6 trials/dB), followed by the 120 dB pulse stimulus 100 ms later. Testing commenced with 10 pulse-alone trials followed by combinations of the prepulse-pulse and null trials, and it terminated with 10 pulse-alone trials. PPI responses were calculated as $\%PPI = [1 - (\text{pre-pulse trials}/\text{startle-only trials})] * 100$.

Radioligand binding and immunohistochemistry of the 5-HT_{2A}R. Binding experiments on mouse brains were conducted as described using 2.3 nM [³H]-ketanserin (NEN Life Sciences, Wellesley, MA) as the radioligand⁵³ with varying concentrations of unlabeled DOI (Sigma-Aldrich) and 75 µg protein from brain. Binding was analyzed by GraphPad Prism (San Diego, CA). The 5-HT_{2A}R immunofluorescence was performed as described⁵³ with a validated 5-HT_{2A}R-specific antibody⁵⁴. Mice were intracardially perfused with PBS followed by 4% PFA in PBS. Brains were harvested, post-fixed overnight in 4% paraformaldehyde, and dehydrated in 30% sucrose. Brains were sectioned at 40 µm by cryostat. Brain sections were washed 3X with 0.4% Triton X-100 in PBS (TX-100/PBS) before incubating for 1 h with blocking buffer (5% normal donkey serum in 0.4%TX-100/PBS). Next, they were incubated for 48 h at 4°C with the anti-5-HT_{2A}R antibody (1:250, #RA24288; Neuromics, Edina, MN). Subsequently sections were washed 3X with 0.1%TX-100/PBS and incubated for 2 h with the secondary antibody (1:1000, donkey anti-rabbit, Alexa Fluor® 594; Jackson ImmunoResearch, West Grove, PA). The sections were imaged under a 20X objective using an Olympus VS120 virtual slide microscope (Olympus, Tokyo, Japan).

Statistics. All statistical analyses were performed with IBM SPSS Statistics 27 programs (IBM, Chicago, IL). The data are presented as means and standard errors of the mean. No sex effects were detected in any experiments. Hence, this variable was collapsed. All data were normally distributed. One- or two-way ANOVA, repeated measures ANOVA (RMANOVA), or analyses of covariance (ANCOVA) were used to analyze the data, followed by Tukey or Bonferroni *post-hoc* analyses. A $p < 0.05$ was considered significant. All results were plotted using GraphPad Prism.

Declarations

Acknowledgements

We thank Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA) for providing us with the new strain of βArr1 mice and Dr. Laura M. Bohn (Scripps Research Institute, Jupiter, FL, USA) for sending us the βArr2 mice. We thank Mr. Mitchell Huffstickler for scoring the representative videos of grooming behaviors in mice and Ms. Jiechun Zhou for breeding, genotyping, and maintaining the βArr1 and βArr2 mice. Some of the behavioral experiments were conducted with equipment and software purchased with a North Carolina Biotechnology Center grant. The results displayed in all figures were plotted using GraphPad Prism 9.1.0 (<https://www.graphpad.com/>). The work was supported by NIDA R37-DA045657 and DARPA [Grant Number DARPA-5822 (HR00112020029)]. Preliminary experiments

from Drs. WCW and BLR were supported by U19-MH082441. The views, opinions, and/or findings contained in this material are those of the authors and should not be interpreted as representing the official views, policies, or endorsement of the Department of Defense or the U.S. Government.

Author contributions

V.N. conducted initial open field studies and some ethological experiments with the β Arr2 mice and he helped to statistically analyze these preliminary data. C.R.M. conducted the behavioral experiments with the β Arr1 and β Arr2 mice and organized the data. R.M.R. oversaw the experiments, statistically analyzed the data, and graphed the results. V.M.P. helped with some of the experiments and he perfused brains for the binding and IHC experiments. Y.T.C. conducted the radioligand binding and immunohistochemical investigations with the β Arr1 and β Arr2 mice. W.C.W. and B.L.R. conceived the experiments, proposed the experimental designs, and wrote the manuscript.

Competing interests

No competing interests for this work by any of the authors.

Data availability

Data that support this study are available from the corresponding authors upon reasonable request.

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Figures

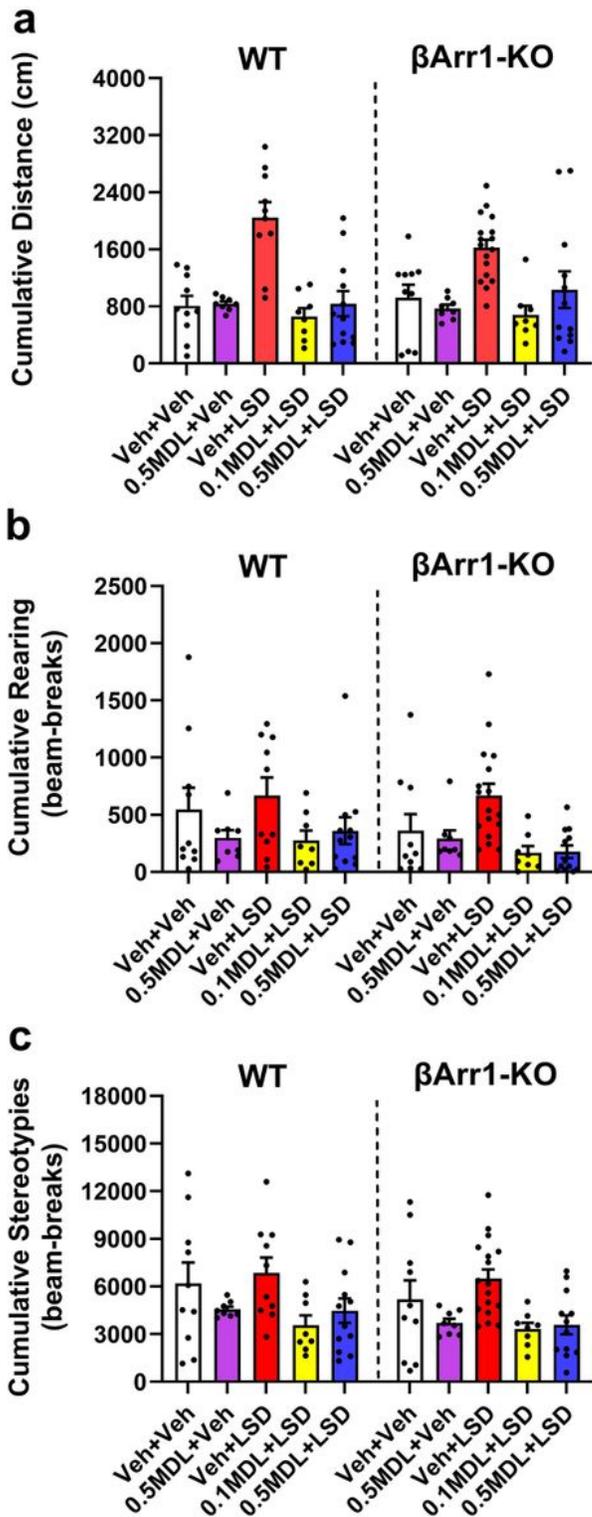


Figure 1

Effects of LSD and MDL100907 on cumulative motor activities in β -arrestin 1 mice. Mice were administered the vehicle or different doses of MDL100907 (MDL) and placed into the open field for 30 min. They were removed injected with the vehicle or 0.3 mg/kg LSD and immediately returned to the test arena for 90 min. The cumulative baseline motor activities (0-30 min) are presented in Supplementary Table S1. A two-way ANOVA failed to identify any significant effects for baseline locomotion; separate

two-way ANOVAs detected significant treatment effects for baseline rearing [$F(4,93)=6.943, p<0.001$] and stereotypical activities [$F(4,93)=7.110, p<0.001$]. To control for these baseline differences in rearing and stereotypy, these LSD post-injection activities were analyzed by ANCOVA. a LSD-stimulated locomotor activities in WT and β Arr1-KO mice. A two-way ANOVA identified a significant treatment effect [$F(4,93)=18.916, p<0.001$]. b LSD-stimulated rearing activities in β Arr1 animals. An ANCOVA failed to find any significant differences. c LSD-stimulated stereotypical activities in β Arr1 mice. An ANCOVA revealed a significant treatment effect [$F(4,92)=7.029, P=0.024$]. N = 8-17 mice/group.

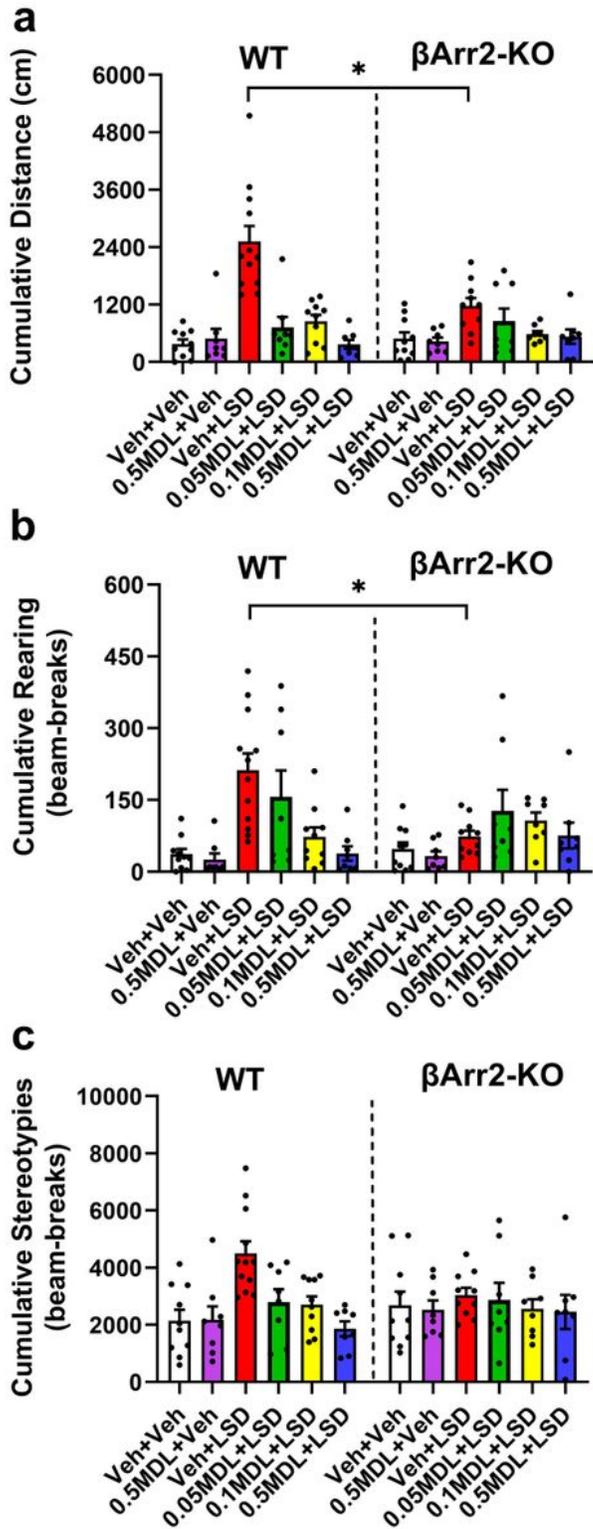


Figure 2

Effects of LSD and MDL100907 on cumulative motor activities in β -arrestin 2 mice. A description of the experimental design is provided in the legend for Figure 1. The cumulative baseline results (0-30 min) are shown in Supplementary Table S2. Two-way ANOVAs failed to identify any significant effects for baseline locomotion, rearing, or stereotypy. a LSD-stimulated locomotor activities in WT and β Arr2-KO subjects. A two-way ANOVA reported a significant treatment effect [$F(5,96)=18.578, p<0.001$] and a significant genotype by treatment interaction [$F(5,96)=5.273, p<0.001$]. b LSD-stimulated rearing activities in β Arr2 animals. A two-way ANOVA observed a significant treatment effect [$F(5,96)=7.150, p<0.001$] and a significant genotype by treatment interaction [$F(5,96)=3.437, p=0.007$]. c LSD-stimulated stereotypical activities in β Arr2 mice. A two-way ANOVA identified a significant treatment effect [$F(5,96)=4.242, p=0.002$]. N = 8-12 mice/group. * $p<0.05$, WT vs. KO.

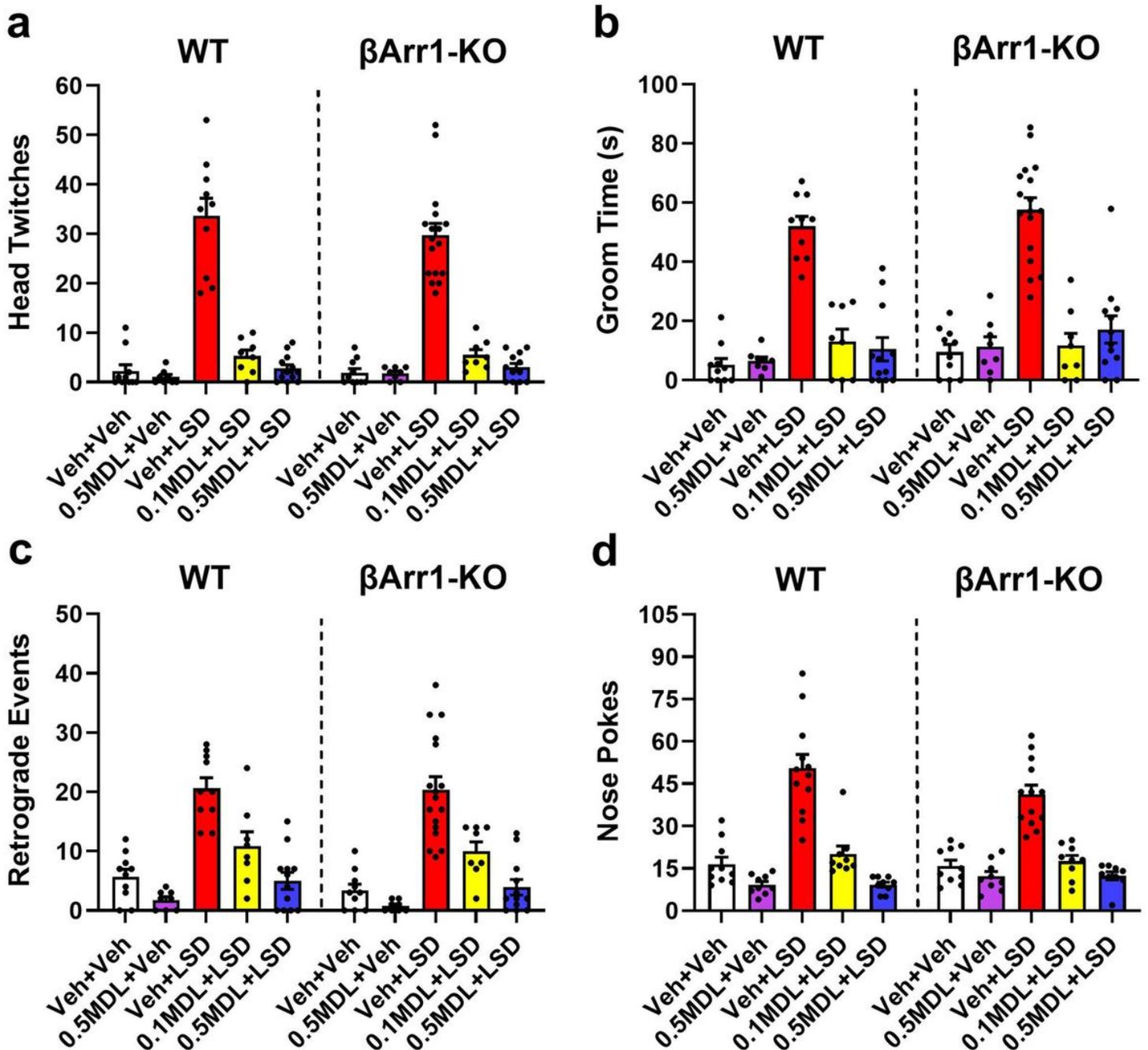


Figure 3

Effects of LSD and MDL100907 on behavioral responses in β -arrestin 1 mice. A description of the experimental design is shown in the Figure 1 legend. The head twitch, grooming, and retrograde walking results represent the first 30 min after injection of LSD in the open field. Nose poking was examined in a 5-choice serial reaction time apparatus (no rewards) with a similar time-course for the vehicle and MDL injections as in the open field, followed by administration of the vehicle and LSD. a LSD-stimulated head twitches in WT and β Arr1-KO mice. A two-way ANOVA revealed a significant treatment effect [$F(4,93)=114.447$, $p<0.001$]. b LSD-stimulated grooming in β Arr1 animals. A two-way ANOVA identified a significant treatment effect [$F(4,93)=61.232$, $p<0.001$]. c LSD-stimulated retrograde walking in β Arr1 subjects. A two-way ANOVA found the main effect of treatment to be significant [$F(4,93)=43.899$, $p<0.001$]. d LSD-stimulated nose poking in WT and β Arr1-KO mice. A two-way ANOVA observed a significant treatment effect [$F(4,89)=60.656$, $p<0.001$]. N = 8-17 mice/group for head twitch, grooming, and retrograde walking; N = 9-13 mice/group for nose-poking.

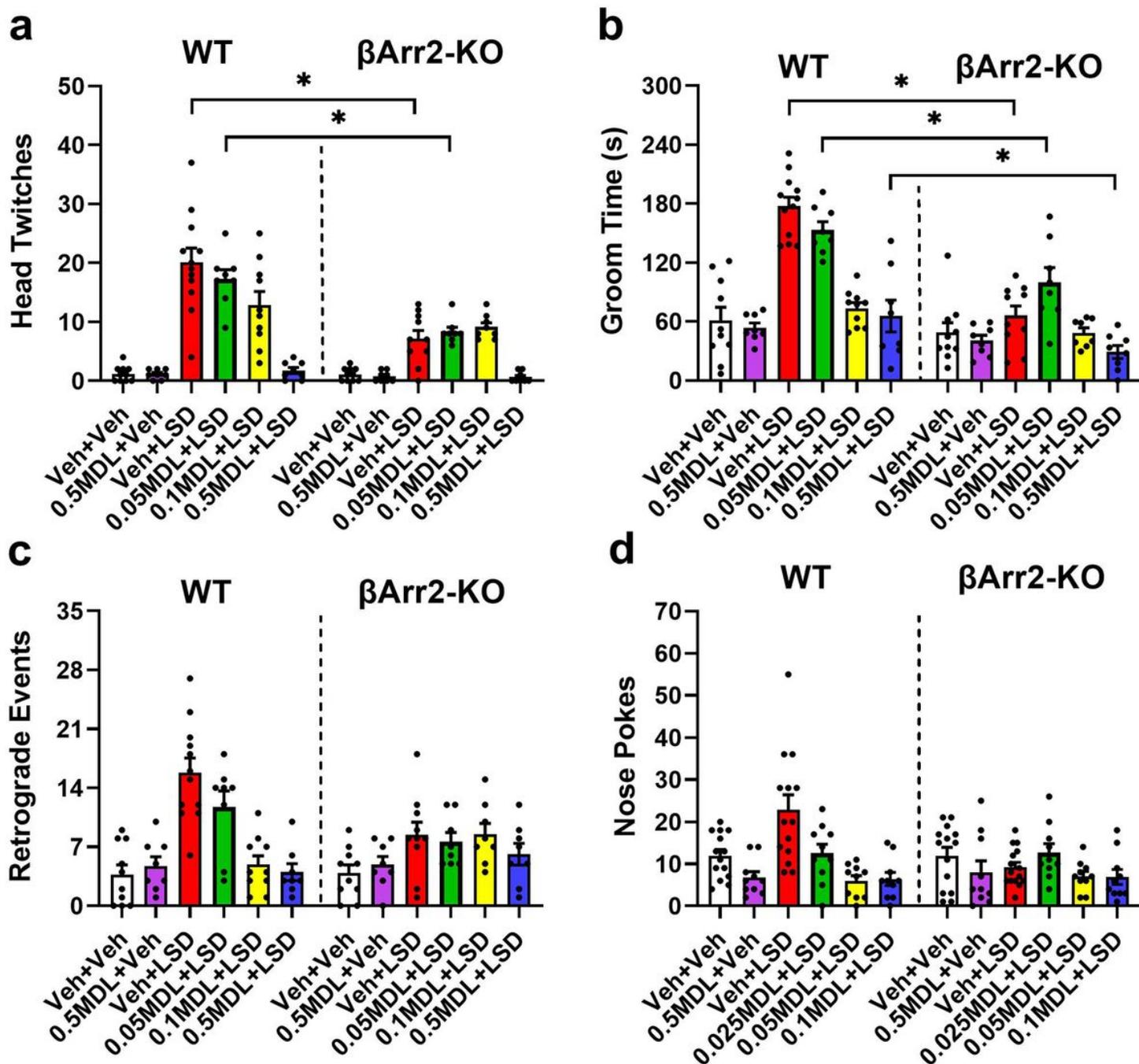


Figure 4

Effects of LSD and MDL100907 on behavioral responses in β -arrestin 2 mice. A description of the experimental design is presented in the Figure 3 legend. a LSD-stimulated head twitches in WT and β Arr2-KO mice. A two-way ANOVA reported significant genotype [$F(1,96)=31.271, p<0.001$] and treatment effects [$F(5,96)=41.567, p<0.001$]; the genotype by treatment interaction was also significant [$F(5,96)=7.734, p<0.001$]. b LSD-stimulated grooming in β Arr2 animals. A two-way ANOVA demonstrated significant genotype [$F(1,96)=51.972, p<0.001$] and treatment effects [$F(5,96)=27.987, p<0.001$]; the genotype by treatment interaction was also significant [$F(5,96)=7.953, p<0.001$]. c LSD-stimulated retrograde walking in β Arr2 subjects. A two-way ANOVA found significant treatment effects [$F(5,96)=13.028, p<0.001$]; the genotype by treatment interaction was also significant [$F(5,96)=5.199,$

p<0.001]. d LSD-stimulated nose poking in WT and β Arr2-KO mice. A two-way ANOVA observed significant treatment effects [F(5,125)=7.512, p<0.001]; the genotype by treatment interaction was also significant [F(5,125)=4.769, p=0.001]. N = 8-12 mice/group for head twitch, grooming, and retrograde walking; N = 10-15 mice/group for nose-poking. *p<0.05, WT vs. KO.

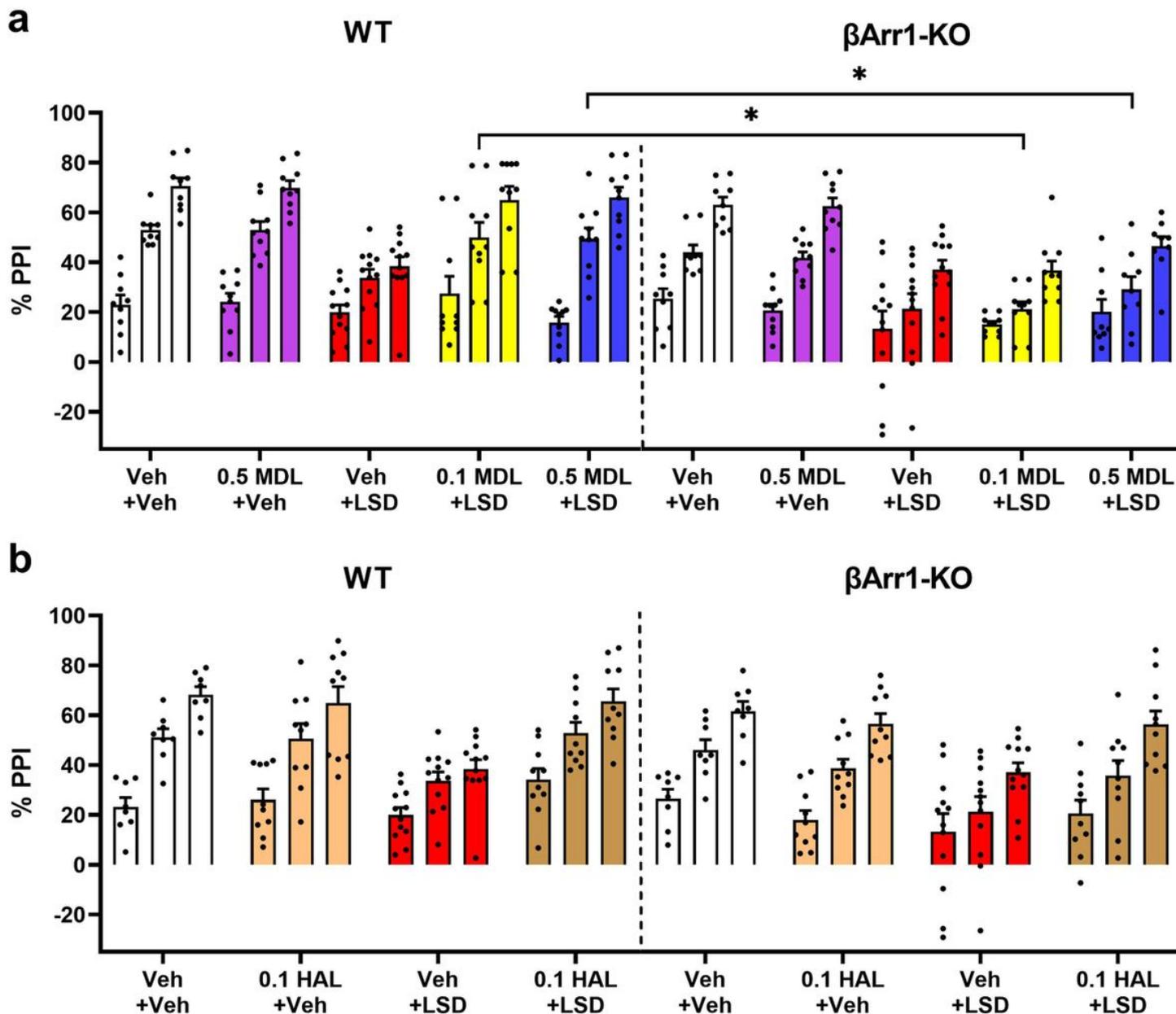


Figure 5

Effects of LSD, MDL100907, and haloperidol on prepulse inhibition in β -arrestin 1 mice. Mice were injected with MDL100907, haloperidol, or the vehicle and administered subsequently the vehicle or LSD prior to testing PPI. a PPI in WT and β Arr1-KO mice treated with MDL or LSD. A RMANOVA found the main effects of prepulse intensity [F(1,91)=487.507, p<0.001], genotype [F(1,91)=25.358, p<0.001], and treatment [F(4,91)=11.435, p<0.001] to be significant. The prepulse intensity by genotype [F(1,91)=9.162, p=0.003], prepulse intensity by treatment [F(4,91)=7.944, p<0.001], genotype by treatment [F(4,91)=2.394, p=0.052], and prepulse intensity by genotype by treatment interactions [F(4,91)=2.611, p=0.041] were

significant. b PPI in WT and β Arr1-KO mice that received haloperidol or LSD. A RMANOVA detected significant main effects for prepulse intensity [$F(1,72)=415.876, p<0.001$], genotype [$F(1,72)=7.563, p=0.008$], and treatment [$F(3,72)=9.591, p<0.001$]. The prepulse intensity by treatment interaction was also significant [$F(3,72)=7.702, p<0.001$]. N = 8-12 mice/group. * $p<0.05$, WT vs. KO.

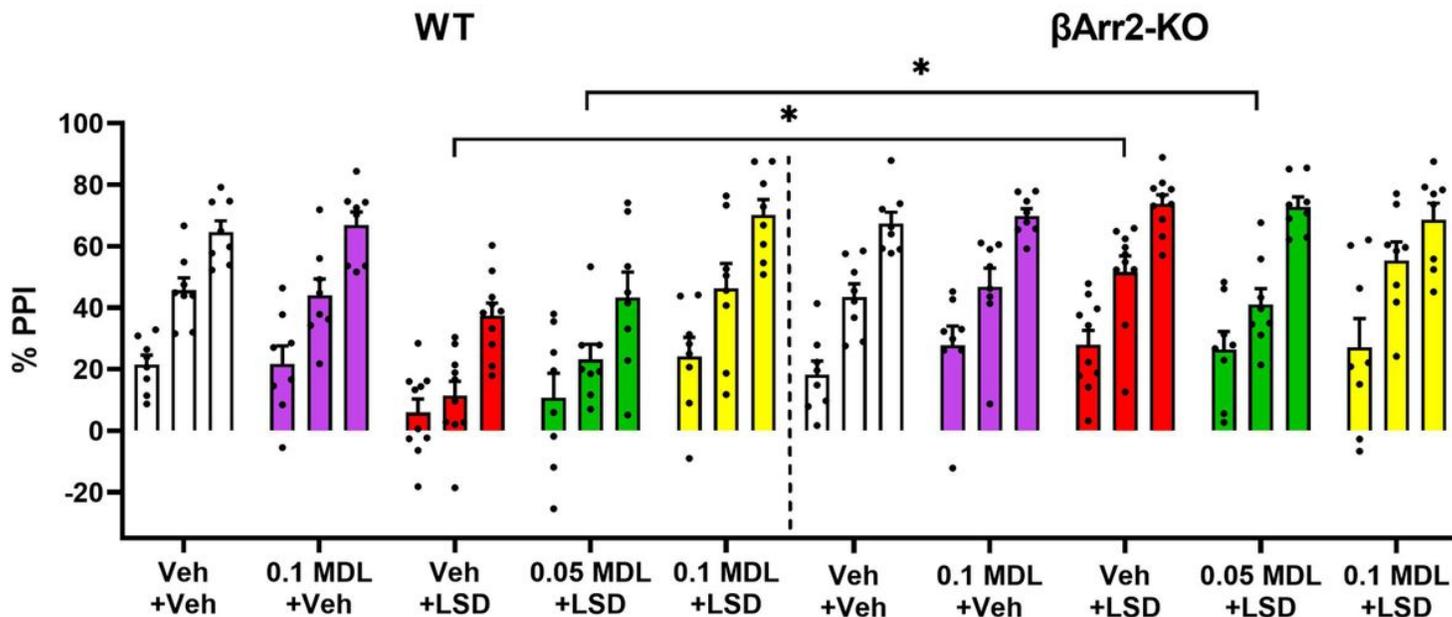


Figure 6

Effects of LSD and MDL100907 on prepulse inhibition in β -arrestin 2 mice. A description of the experimental design is provided in the Figure 5 legend. PPI in WT and β Arr2-KO mice treated with MDL or LSD. A RMANOVA the main effects of prepulse intensity [$F(1,74)=580.044, p<0.001$], genotype [$F(1,74)=18.823, p<0.001$], and treatment [$F(4,74)=3.953, p=0.006$] to be significant; the genotype by treatment interaction [$F(4,74)=5.660, p<0.001$] was also significant. N = 8-10 mice/group. * $p<0.05$, WT vs. KO.

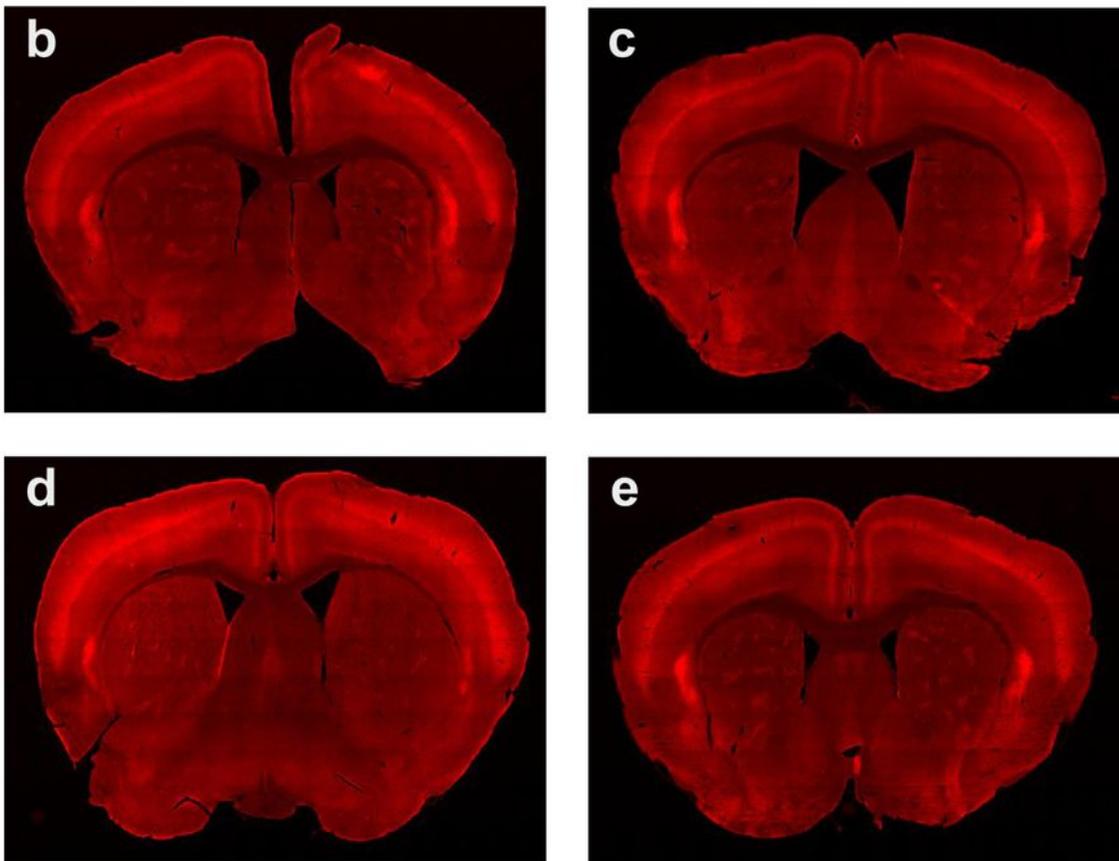
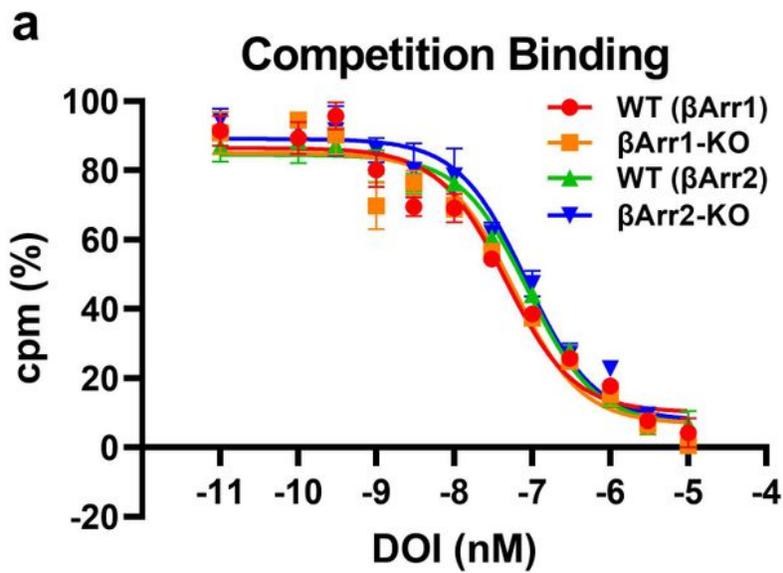


Figure 7

Radioligand binding and immunohistochemistry of 5-HT_{2A}Rs in β Arr1 and β Arr2 mice. a Competition binding with [³H]-ketanserin using membranes from β Arr1 and β Arr2 brains. The K_d values for binding were 21.3, 26.8, 40.8, and 38.7 nM from WT and β Arr1-KO, and WT and β Arr2-KO mice, respectively. N = 3 mice/genotype. b-e Representative 5-HT_{2A}R immunofluorescence in coronal brain sections from respective WT and β Arr1-KO mice (top), and WT and β Arr2-KO (bottom) animals.

Supplementary Files

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